Diversity of *Potamolithus* (Littorinimorpha, Truncatelloidea) in a high-diversity spot for troglobites in southeastern Brazil: role of habitat fragmentation in the origin of subterranean fauna, and conservation status

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Abstract

The Alto Ribeira karst area, southeastern Brazil, is a high-diversity area for troglobites. Three species of freshwater gastropods *Potamolithus* occur in the area: *P. ribeirensis*, only found in epigean waters at the Iporanga and Ribeira rivers; *P. troglobius*, which is endemic to the Areias cave system; and *P. karsticus*, a troglophilic species from Calcário Branco Cave and an epigean stream nearby. We investigated their distribution based on shell morphology and internal anatomy of epigean species, troglophilic populations, and troglobitic species. Distribution patterns of *Potamolithus* were compared to those of other aquatic taxa from the region (such as crustaceans and fishes). Besides the three species already described for the region, we recorded 12 additional ones, for a total of 15 species/morphs (six troglobites, seven troglophiles, and two epigean). *Potamolithus* spp. are restricted to micro-basins and/or caves, showing small areas of distribution and probably a high degree of endemism. Geomorphology (irregular landscape, with limestone outcrops intercalated with insoluble rocks, which probably act as geographic barriers for cave populations), paleoclimatic evidence, and ecological/biological factors, such as the low degree of mobility of these gastropods (sedentary habit), explain the distributional patterns. We observed troglomorphisms such as reduction/absence of eyes and pigmentation (body and periostracum), and a coiled intestine. Apparently, there is no cause-and-effect between miniaturization and intestine coiling for *Potamolithus*, in contrast to observations for other cave snails. *Potamolithus* snails are threatened in the region due to water pollution, uncontrolled tourism, and overcollection.

Introduction

Neritimorpha gastropods, and mainly neogastropods, have been recorded in the subterranean (hypogean) realm (e.g., *Theodoxus subterrelictus* Schütt, 1963; *Georissa papuana* Bernasconi, 1995). Among neogastropods, truncatelloids comprise 97% of the troglobitic species (with source populations restricted to the hypogean environment; Trajano 2012) (Bole and Velkovrh 1986). Subterranean truncatelloids also include troglophilic populations (subterranean source populations genetically connected with epigean populations of the same species by individuals commuting between these environments; Trajano 2012).

In general, troglobites may be distinguished by the presence of characteristics related to isolation in the hypogean environment, the so-called troglomorphisms. The most ubiquitous troglomorphisms are a reduction, or complete loss, of the eyes and melanic pigmentation, observed in many subterranean truncatelloids throughout the world (e.g., Boeters 1979). Other frequent specializations reported for troglobitic truncatelloids are miniaturization, and, as a consequence, the complex coiling of the intestine and a lack, or reduction, of the ctenidium and the semen receptacle (Hershler and Holsinger 1990). This miniaturization is proposed as an adaptive characteristic to environments with poor food supplies (see Sanders and Allen 1973, Hershler and Longley 1986), or as an ancestral characteristic present in epigean relatives (exaptation, *sensu* Arnold 1994).

Truncatelloids are very common in caves in Europe, North America, Africa, Japan, Australia, and New Zealand (Hershler and Longley 1986, Angelov 2000, Ponder et al. 2005); there are also records from the phreatic waters of the Toscana region, Italy (Bodon et al. 1996). In South America, a troglomorphic species, *Andespyrgus sketi* Hershler & Velkovrh, 1993, was reported from caves in Ecuador and Colombia, and several species of *Potamolithus* Pilsbry, 1896 have been found in caves from southeastern Brazil (Simone and Moracchioli 1994, Bichuette 1998).

The genus *Potamolithus* is characterized by a tiny, oval to rounded shell, with a prosocline, rounded to oval aperture; the shell whorls are arched and the last one is much larger than the others (*sensu* Wenz 1938). According to the World Register of Marine Species (WoRMS Editorial Board 2017), the genus comprises 18 species, which occur in the freshwaters of meridional South America, mainly in the Ribeira, Itajaí-açú, and Jacuhy Rivers in southern Brazil, and the Uruguay River, part of the Paraná and Río de la Plata drainage systems, with one species in Patagonia (de Lucía and Gutiérrez-Gregoric 2017a); nine of these species occur in Brazil. de Lucía and Gutiérrez-Gregoric (2017b) redescribed *Potamolithus supersulcatus* Pilsbry, 1896 for Argentina, and concluded that the genus possesses 31 valid species, which is in contrast to other databases (e.g., WoRMS Editorial Board 2017), reinforcing the problem of

inconsistent data for this group. For example, *Potamolithus* was traditionally classified, based on morphology, as Lithoglyphidae (Davis and Pons-da-Silva 1984) and as Hydrobiidae (e.g., Simone 2006). However, molecular studies indicated a position within the Tateidae (Wilke et al. 2013), a much smaller family with 32 genera, distributed in Australasia/Oceania and the Neotropics (Wilke et al. 2013, Haase and Zielske 2015). Considering the possibility of conflict with molecular phylogenies and morphology-based classifications, as recently shown for some Tateidae (Becker et al. 2016), we choose not to take any position regarding the familial status of *Potamolithus* until all the evidence corroborates one of these, or different, taxonomic hypotheses. Comparisons are made with other subterranean truncatelloids.

The Alto Ribeira karst area, in São Paulo State, southeast Brazil, is a high-diversity spot for troglobites (Trajano et al. 2016). So far, three species of *Potamolithus* have been described for this area: *Potamolithus ribeirensis* Pilsbry, 1911, only found in epigean waters of the Iporanga and Ribeira rivers; *Potamolithus troglobius* Simone & Moracchioli, 1994, from the Areias cave system (the first troglobitic mollusk from Brazil); and *Potamolithus karsticus* Simone & Moracchioli, a troglophilic species, from Calcário Branco cave and an epigean stream nearby. Other Brazilian troglobitic aquatic gastropods include *Spiripockia punctata* Simone, 1994 (Truncatelloidea: Pomatiopsidae), a new genus and species described from Serra do Ramalho karst area, Bahia State, northeast Brazil, and an undetermined species, possibly also of the genus *Spiripockia* Simone, 2012, from Serra da Bodoquena karst area, Mato Grosso do Sul, southwest Brazil.

The paleoclimatic model (Barr 1968), based on alternating humid and dry phases of glacial cycles as periods of colonization of subterranean habitats followed by isolation and differentiation respectively, in the habitats in which troglobites originate, has been proposed for tropical areas, particularly Brazil, since the early 1990s (Trajano 1995, 2001, Trajano and Britski 1992, Bichuette et al. 2015). Geomorphological and paleontological evidences point to the existence of a corridor of open vegetation along the Rio Ribeira valley, southeastern Brazil, during the last glacial phase (Ab'Sáber 1981). This region is currently covered by Atlantic rainforest. Dating of speleothems from Santa Catarina, southern Brazil (Cruz et al. 2005) corroborates the hypothesis of dry glacial and humid interglacial phases in SE-S Brazil. Therefore, in the Alto Ribeira karst area, the interglacial phases would be times of colonization of caves and establishment of troglophilic populations, alternating with glacial dry phases when forests would be substituted by open vegetation and the epigean drainage would be disrupted, isolating those troglophilic populations in the subterranean environment (Trajano and Britski 1992, Trajano 1995). This model was recently proposed by Fernandes et al. (2013) to explain the diversity of troglobitic anomuran crustaceans, genus *Aegla* Leach, 1820, in the Alto Ribeira.

Subterranean systems are threatened throughout the world and much-needed conservation policies depend, among other factors, on knowledge of the ecology, biology, and behavior of subterranean species. Because truncatelloids are small organisms with low mobility, but with species in different environments all over the world, there is great interest in their biogeography, comparative ecology, behavior, and physiology

(Davis et al. 1982). However, these studies depend on the recognition and delimitation of species, as accurately as possible with the available data, as a basis for systematics analysis and for proposals for phylogenetic hypotheses. The few existing studies on Brazilian freshwater truncatelloids have focused mainly on the description and distribution of a few species (Pilsbry 1911, Malek 1983, Pons-da-Silva 1993, Simone and Moracchioli 1994) and the ecology and behavior of cave *Potamolithus* (Bichuette and Trajano 1999, 2003).

As part of a broader investigation on truncatelloids from the Alto Ribeira karst area (Bichuette 1998), that included population ecology (Bichuette and Trajano 2003) and behavior (Bichuette and Trajano 1999, Bichuette and Menna-Barreto 2005), the main cave systems and associated epigean (surface) streams were surveyed and a comparative taxonomic study, based on shell morphology and external and internal anatomy, was performed. We present the results of this study, as a contribution to knowledge of the diversity of subterranean aquatic gastropods and morphological specializations of troglobites, by comparing epigean species, troglophilic populations, and troglobitic species. Furthermore, analyses of distribution patterns, exploring their causes/factors, were compared to other aquatic taxa, such as the anomuran *Aegla* crustacean (Decapoda).

Materials and methods

Study area

The study area is situated in the Alto Ribeira karst area, São Paulo State, southeastern Brazil. It is geologically inserted in the Açungui Group, composed of Upper Precambrian metasedimentary rocks (Karmann and Sànchez 1979). Four NE-SW limestone outcrops extend over 20 km, with widths varying from 1 to 8 km, alternate with insoluble detrital rocks, such as phyllites (Trajano et al. 2016) (Figure 1).

The Alto Ribeira river valley is located in the transition between the Tropical Atlantic and Araucaria Forest domains (Ab'Sáber 1977) (Figure 2). The climate in the study area is type "Cfa", which is defined as tropical wet to temperate humid without a dry season. Total precipitation is around 1,500 mm and precipitation in the driest and coolest month (July) is more than 30 mm. Temperatures are above 22 °C in the warmest month (January) and below 4 °C in the coolest month; mean annual temperature (as generally observed deep inside caves) is around 19–20 °C in the Parque Estadual Turístico do Alto Ribeira (PETAR), and 17–18 °C in the upper Parque Estadual Intervales (PEI), where collections were carried out (Köeppen 1948). This area is mostly covered by perennial subtropical humid forest (Hueck 1972), with typical well-oxygenated headwater streams, with rocky beds and rapids alternating with some soft-bottomed pools, which cross the rock outcrops (Figure 2).

Faunistic surveys were conducted in epigean and cave stream reaches in two continuous regions in the Alto Ribeira karst area, mostly protected by state parks: PETAR
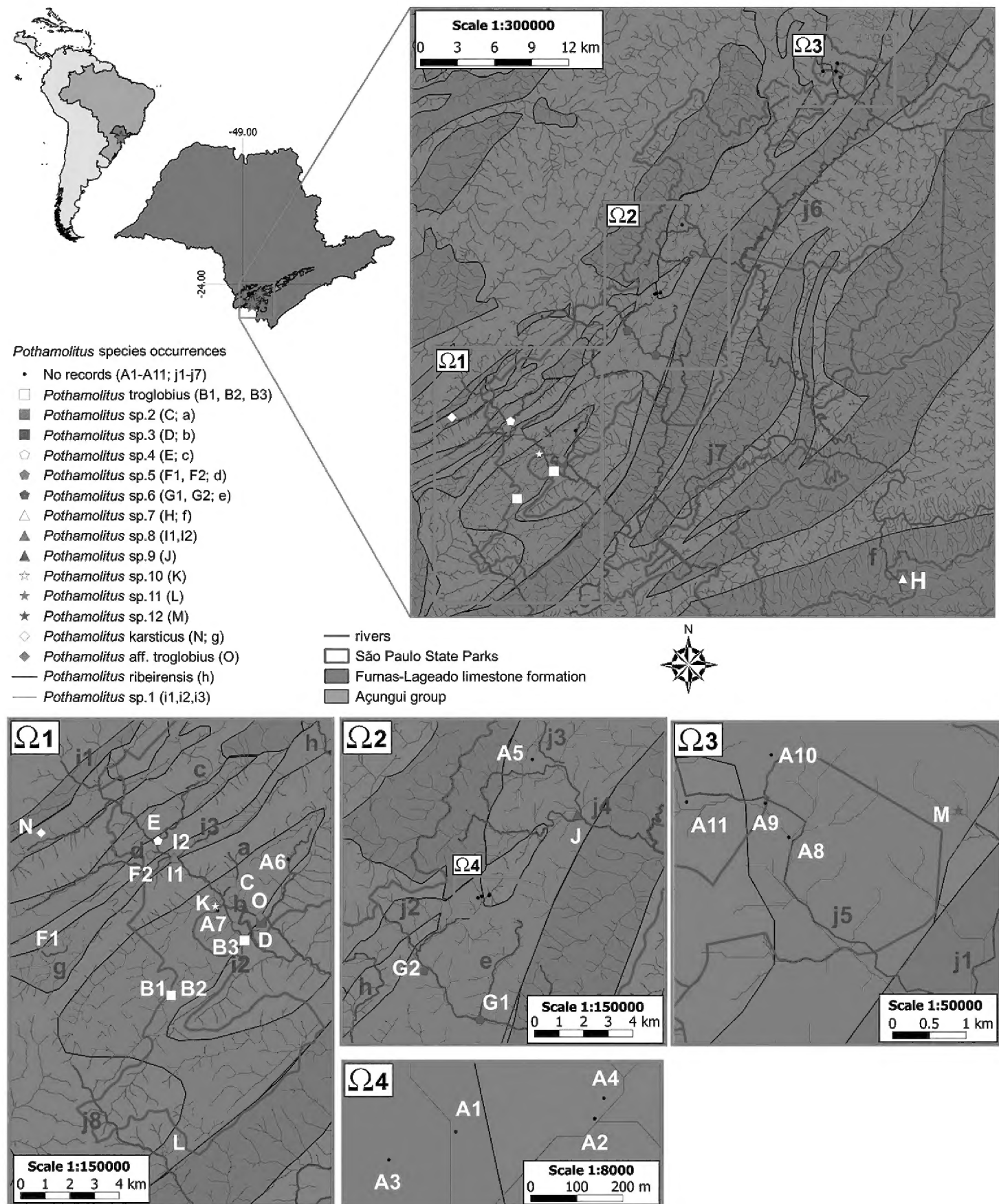
Figure 1. Map showing the surveyed localities (basins, microbasins and caves) from Alto Ribeira karst area, Southeastern Brazil. Some localities are approximated (*) (Author: DM von Schimonsky). **Caves**: A1 – Aranhas, A2 – Chapéu Mirim I, A3 – Chapéu, A4 – Chapéu Mirim II, A5 – Temimina II, A6 – Gurutuva, A7 – Córrego Seco, A8 – Fendão, A9 – Paiva, A10 – Jane Mansfield, A11 – Minotauro; B1 – Areias de Cima, B2 – Areias de Baixo, B3 – Ressurgência das Areias de Água Quente; C – Ouro Grosso; D – Alambari de Baixo; E – Água Suja; F1 – Pérolas, F2 – Santana; G1 – Casa de Pedra, G2 – Água Sumida; H – Tapagem; I1 – Morro Preto, I2 – Couto; J – Pescaria; K* – Betari de Baixo; L – Jeremias; M – Colorida; N – Calcário Branco; O – Alambari de Cima. **Epigean streams**: a – Ouro Grosso; b – Alambari; c – Água Suja; d – Roncador; e – Maximiano; f – Ostras; g – Calcário Branco; h – Iporanga; i1 – Betari, i2 – Água Quente, i3 – Morro Preto; j1 – Bocaina, j2 – Espírito Santo, j3 – Temimina, j4 – Pescaria, j5 – Lageado, j6 – Pilões, j7 – Ribeira, j8 – Cutia de Cima.
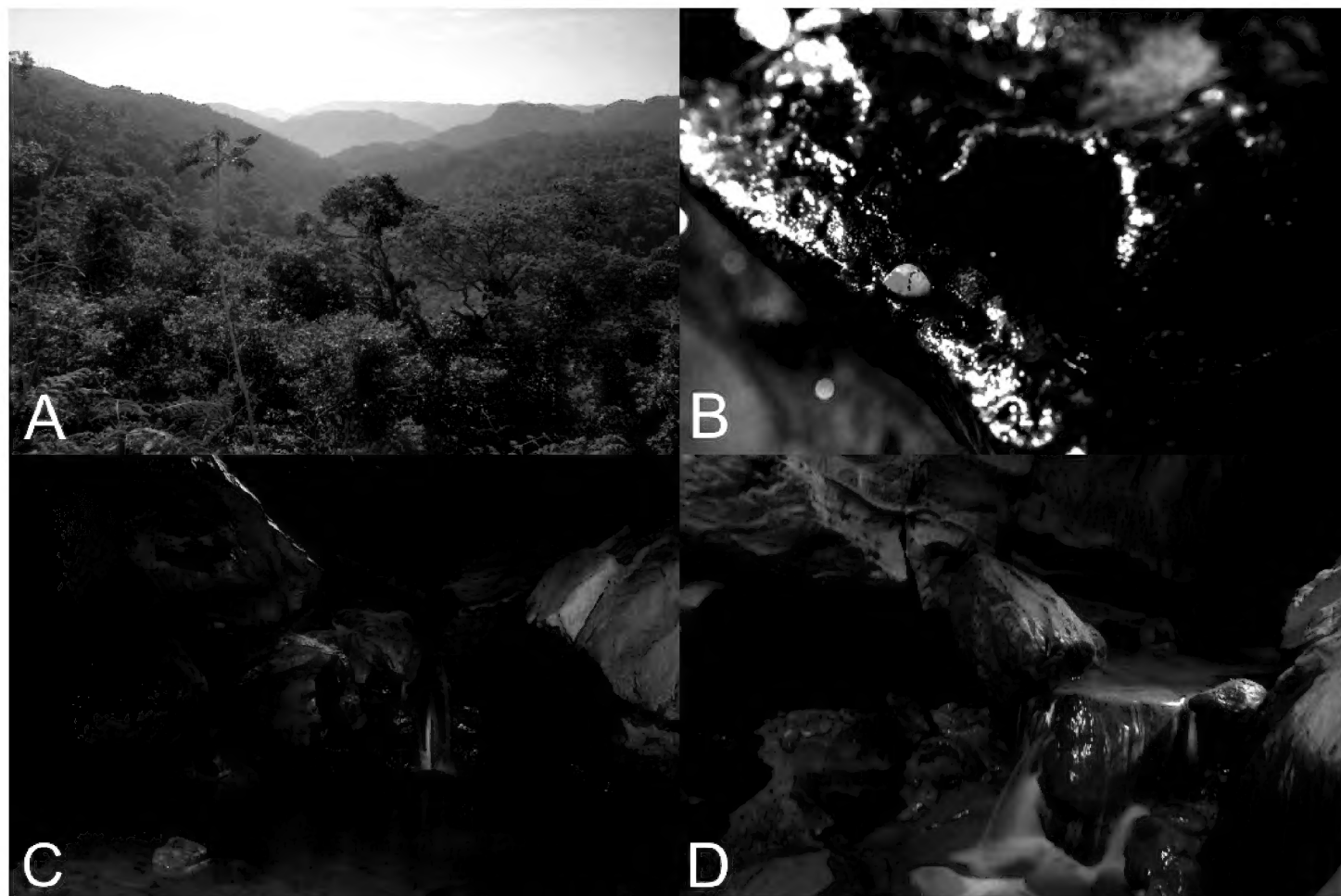
Figure 2. Landscape (Atlantic Rainforest) (**A**), *Potamolithus* sp. 5 in natural habitat, Santana cave (**B**) and subterranean streams (**C, D**) from Alto Ribeira karst area, Southeastern Brazil. Photographs: PP Rizzato (**A, C, D**), A Gambarini (**B**).

and PEI. Altitudes vary from 300 to 1,000 m in PETAR, which is crossed by four limestone outcrops. One of these outcrops bifurcates and extends toward the northeast, crossing PEI, where altitudes reach up to 1,200 m.

We visited 33 caves and 10 epigean streams in PETAR and four caves and two epigean streams in PEI (Figure 1). We also collected in the Tapagem Cave (a partially touristic cave, also known as Caverna do Diabo - Devil´s Cave) and the epigean stream that sinks into it, a relatively isolated karst area in the middle of the Rio Ribeira valley, protected by the Parque Estadual Caverna do Diabo (PECD). The survey areas were located in four basins, or micro-basins (Figure 1).

The caves (from northeast to southwest) are shown in Figure 1: PEI - Fendão, Paiva, Jane Mansfield, Minotauro, and Colorida; PETAR - Temimina II, Pescaria, Aranhas, Chapéu, Chapéu Mirim I, Chapéu Mirim II, Água Sumida, Casa de Pedra, Calcário Branco, Água Suja, Santana, Pérolas, Morro Preto, Couto, Córrego Seco, Alambari de Cima, Alambari de Baixo, Gurutuva, Ouro Grosso, Areias de Cima, Areias de Baixo, Ressurgência das Areias de Água Quente, Betari de Baixo, and Jeremias; PECD - Tapagem.

Rivers, separated by micro-basins, are also shown in Figure 1: Pilões micro-basin - Pilões, Lageado, Pescaria, Temimina, and Bocaina; Iporanga micro-basin - Iporanga, Maximiano, and Espírito Santo; Betari micro-basin – Betari, Calcário Branco, Água Suja, Roncador, Morro Preto, Ouro Grosso, Alambari, and Água Quente; Ostras and Cutia de Cima micro-basins.

Methods

Systematic field trips to the Alto Ribeira karst area were carried out during 11 months between 1996 and 1997 (Bichuette and Trajano 2003); additional collections were done in 2008, 2009, 2010 and 2013 (four samplings, one per year). *Potamolithus* specimens were collected by hand after visual inspection of the substrate, mostly under boulders, pebbles, and other submerged objects, and by straining of thin substrates, such as sand and clay. Specimens were fixed in 70% ethanol and brought to the laboratory for morphological and taxonomic study. Identification at the genus level was confirmed with the collaboration of LRL Simone (Seção de Moluscos, Museu de Zoologia da Universidade de São Paulo, MZUSP).

For a preliminary separation of species/morphs, we considered the following characters in samples containing between 15 and 82 specimens: shape of intestine, observed by transparency in an abapertural view (slightly curved to left; slightly curved to right; markedly curved to left; two marked curves, a broad fold to left; a constricted fold to left) (Figure 3); pattern of body (mantle) pigmentation; presence *versus* absence of eyes; shell shape (fusiform; globose; fusiform-globose); shell dimensions (height – from the apex to the base, following the columellar axis, and width – the maximum measurement at a right angle to the columellar axis); and periostracum coloration observed in live individuals (dark brown, brown, pale yellow, white, translucent). Shell measurements were taken using digital calipers with 0.01 mm precision; we analyzed only adult individuals (smaller individuals that showed mature gonads – see Bichuette and Trajano 2003). We followed the anatomical nomenclature from Simone (2006).

To illustrate the shell opening, shape, and ornamentation, one or two individuals per population were studied under a scanning electron microscope (SEM) (Zeiss electron microscope) and through images acquired via LAS software (Leica Application Suite v3.7). Criteria for distinction between troglobites and troglophiles, according to the Schiner-Racovitza system, were used as in Trajano and Carvalho (2017). The absence of records in the epigean environment allied to troglomorphic characters (such as translucent periostracum and absence of eyes) were considered for troglobite status; for troglophile status, we considered the occurrence of well-established (source) populations in both epigean and hypogean environments, indicated by the presence of many individuals of all size/age classes deep inside caves throughout the annual cycle.

Examined material was deposited at the Museu de Zoologia, Universidade de São Paulo (MZUSP) and the Laboratório de Estudos Subterrâneos, Universidade Federal de São Carlos (LES).

Data analysis

To detect statistical differences among individuals from each OTU (Operational Taxonomic Unit - Sokal and Sneath 1963), the shell sizes (adults) were visually compared using boxplot graphs. A variance analysis (One Way ANOVA), followed by
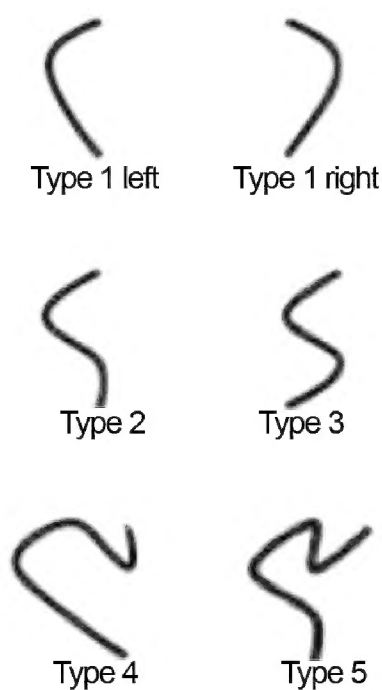
Figure 3. Schematic intestine forms observed in *Potamolithus* species from Alto Ribeira karst area, Southeastern Brazil. Abapertural view.

Dunn's post-hoc test, was performed using the software PAST version 3.09 (Hammer et al. 2001). Before the analysis, data were tested in relation to normality and homogeneity of variances.

Species concept adopted

The concept of a species is one of the most complicated and debatable problems in biology. For several decades now, hundreds of publications have focused on this subject but no consensus has been achieved, and probably never will be (see Wheeler and Meier 2000, for a debate on the theme). Herein we adopted the Operational Taxonomic Unit (OTU; Sokal and Sneath 1963), which most taxonomists use in their daily task of recognizing, characterizing, and describing "species" and most inclusive clades. This is the first, and necessary, step for phylogenetic studies, as well as ecological studies for conservation, among others. Describing biodiversity is urgent for several reasons, and the OTU approach is not only a practical way to begin, but has a sound theoretical basis.

In our approach, OTUs are a spin-off from the evolutionary species ("An evolutionary species is an entity composed of organisms that maintains its identity from other such entities through time and over space and that has its own independent evolutionary fate and historical tendencies"), or species-as-lineage concepts (Wiley and Mayden 2000). According to these authors, if lineages are independent, at the empirical level we can expect to eventually discover differences among them. Such differences are expressed as character states indicating the lineages' individuality (not to be confused with statements of similarity).

Therefore, the species as recognized and characterized below are lineages distinguished by sets of diagnostic traits (that may be apomorphic or plesiomorphic, which is not relevant in our approach).

Results

Potamolithus gastropods are widely distributed in the Alto Ribeira karst area, at least in São Paulo State, at altitudes lower than 800 m. In epigean rivers, these animals occur under pebbles, boulders, or branches with relatively smooth surfaces. In the hypogean environment, they occur both under and over pebbles and rocky blocks with smooth surfaces.

After a collecting effort of ca. 60 days of fieldwork, besides the three species of *Potamolithus* previously described, we recorded 12 additional distinguished morphs (OTUs) (Table 1 and Figures 4 to 8). The occurrence of one species per cave (for most records) and the mosaic distribution of character states in the sampled populations (see below) support their treatment as separate morphs (OTUs), which we refer to as unnamed *Potamolithus* spp. We numbered the species in increasing order according to their status in the Schiner-Racovitza system (see Methods). Two of the species were only sampled in epigean streams (referred to as epigean), seven were from source populations in both epigean and subterranean streams (troglophiles), and six were found exclusively in caves (troglobites), showing troglomorphisms. We could not find consistent differences separating the Alambari de Cima population from *P. troglobius*, and we, therefore, conservatively treated the former as *Potamolithus* aff. *troglobius*. Intraspecific variability in the presence *versus* absence of eyes, and pigmentation of the mantle and
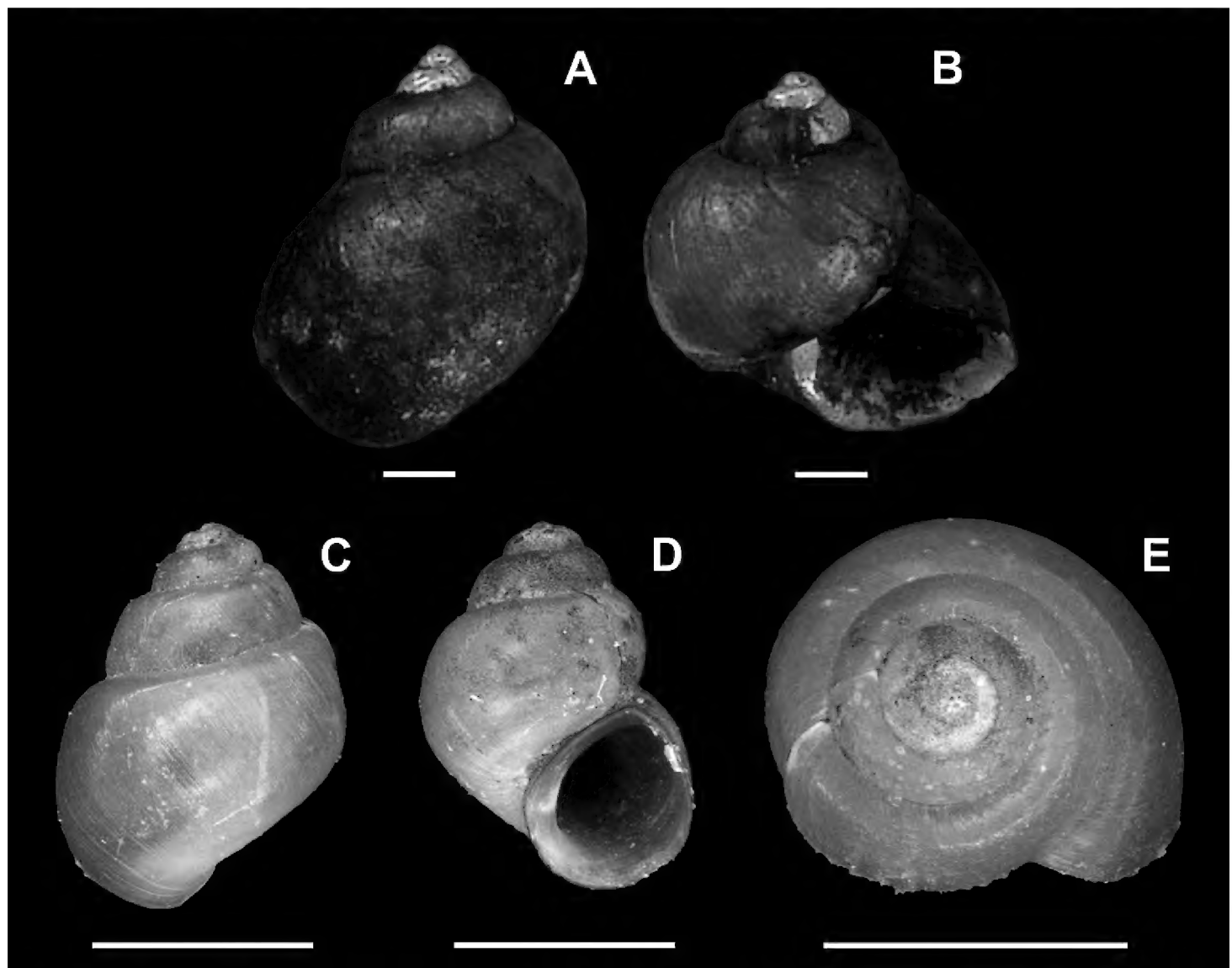

Figure 4. *Potamolithus ribeirensis -* **A** (dorsal view) **B** (apertural view); *Potamolithus* sp. 1 **C** (dorsal view) **D** (apertural view) **E** (apical view). Scale bars: 1mm. (Photographs: LBR Fernandes).
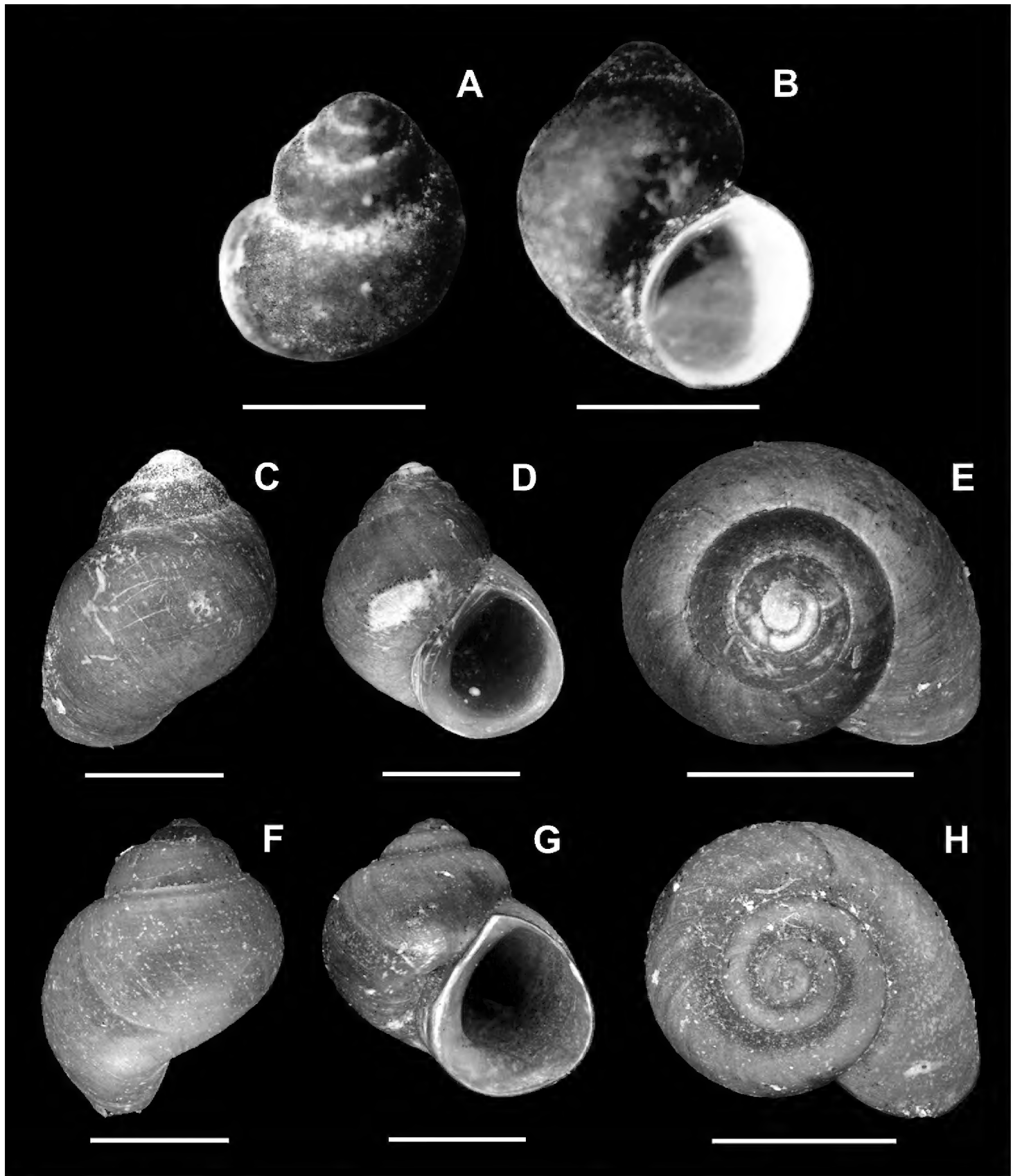
Figure 5. *Potamolithus karsticus* - **A** (dorsal view) **B** (apertural view); *Potamolithus* sp. 2 **C** (dorsal view) **D** (apertural view) **E** (apical view); *Potamolithus* sp. 3 **F** (dorsal view) **G** (apertural view) **H** (apical view). Scale bars: 1mm. (Photographs: LBR Fernandes).

the periostracum, were observed for *Potamolithus* spp. 5 (troglophile), 8, and 9 (troglobites) (Table 1, Figures 4–8).

Figures 9 and 10 show, respectively, the distributions of frequencies of shell height and width, comparing the epigean, troglophilic, and troglobitic samples. SEM images showed an absence of ornamentation on both proto- and teleoconch.

The one-way ANOVA analyses revealed a significant difference between the shell sizes (height) of *Potamolithus* species (F = 48.12; df1 = 15; df2 = 671; p = 8.211E-96).
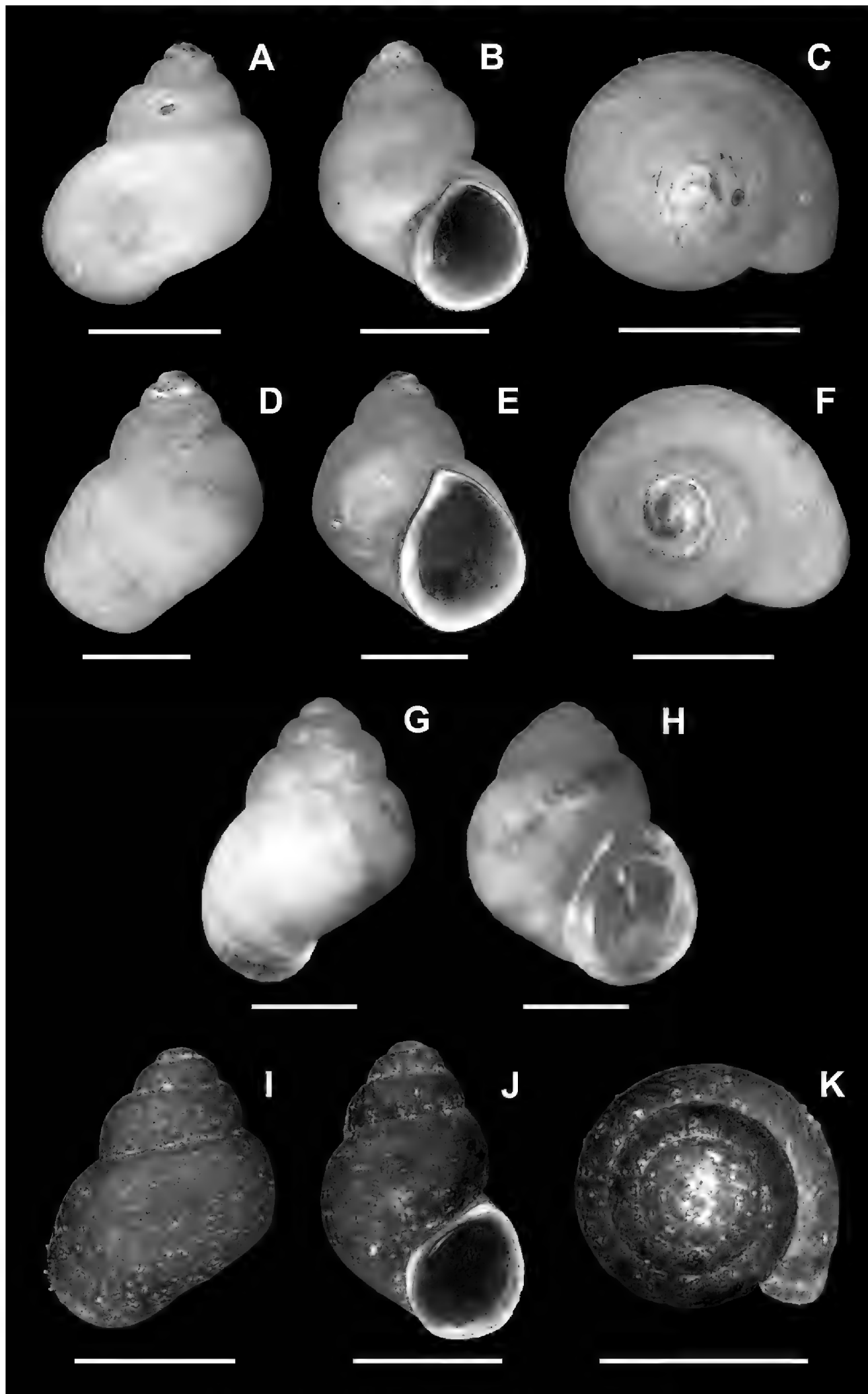
Figure 6. *Potamolithus* sp. 4 **A** (dorsal view) **B** (apertural view) **C** (apical view); *Potamolithus* sp. 5 **D** (dorsal view) **E** (apertural view) **F** (apical view); *Potamolithus* sp. 6 **G** (dorsal view) **H** (apertural view); *Potamolithus* sp. 7 **I** (dorsal view) **J** (apertural view) **K** (apical view). Scale bars: 1 mm. (Photographs: LBR Fernandes).
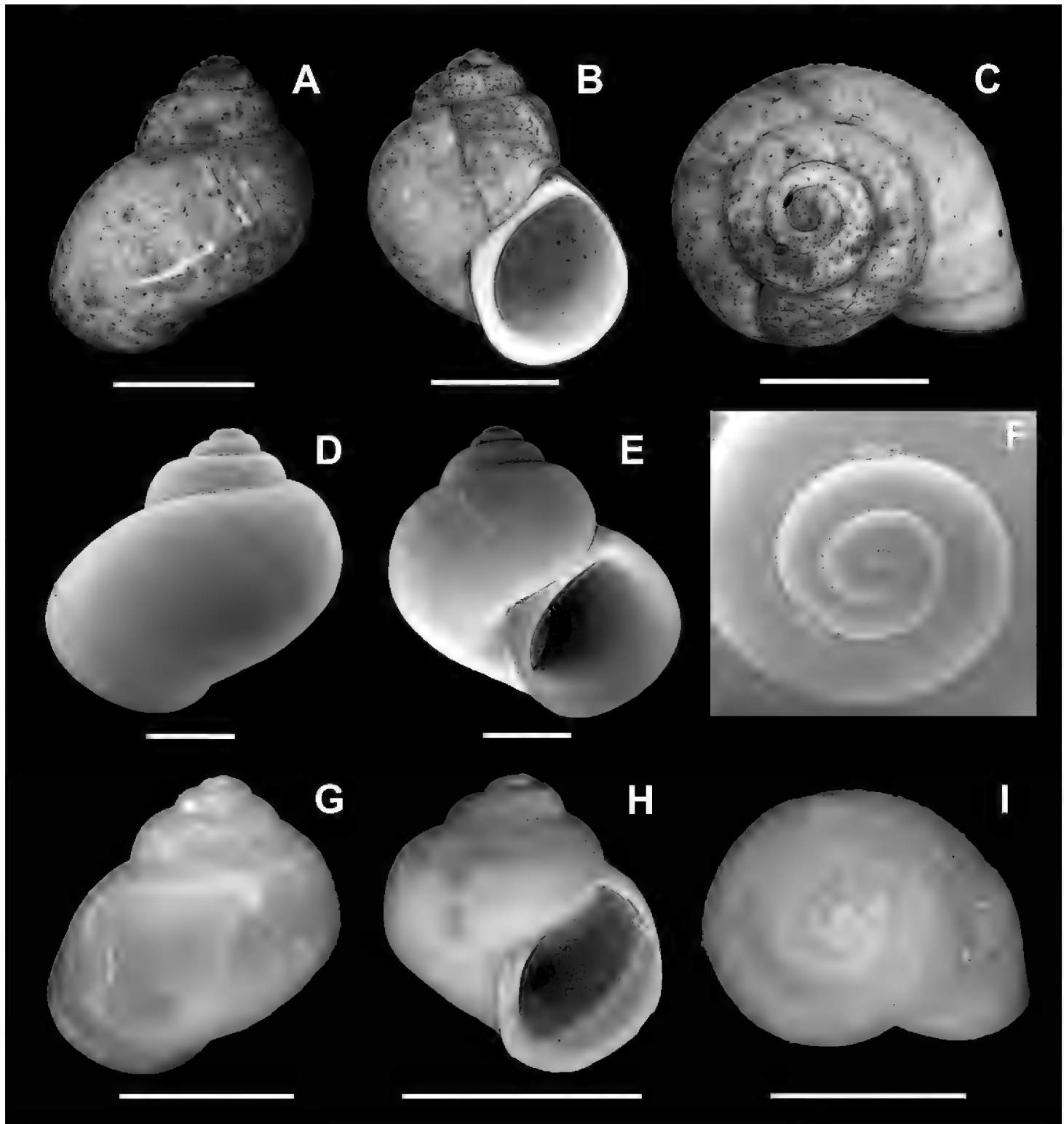
Figure 7. *Potamolithus troglobius* **A** (dorsal view) **B** (apertural view) **C** (apical view); *Potamolithus* aff. *troglobius* **D** (dorsal view) **E** (apertural view) **F** (apical view); *Potamolithus* sp. 8 **G** (dorsal view) **H** (apertural view) **I** (apical view). Scale bars: 1mm. (Photographs: LBR Fernandes).

The post-hoc analyses (Dunn´s test) (Table 2) showed that smaller sizes, especially in the troglobite from Colorida cave (*Potamolithus* sp. 12), were also observed in troglophilic ones (e.g. *P. karsticus*, *Potamolithus* spp. 4 and 7) and in the epigean population from the Betari basin (*Potamolithus* sp. 1). On the other hand, the troglobitic gastropods from Pescaria cave, *Potamolithus* sp. 9, reached larger sizes, only surpassed by the epigean *P. ribeirensis* (Figures 9 and 10, Table 2 for statistical results).

As mentioned, there is a mosaic of character states, with almost all species differing from the others by at least one state. The only exceptions were *P. troglobius* and the population from Alambari de Cima cave; for this reason, the latter was treated as
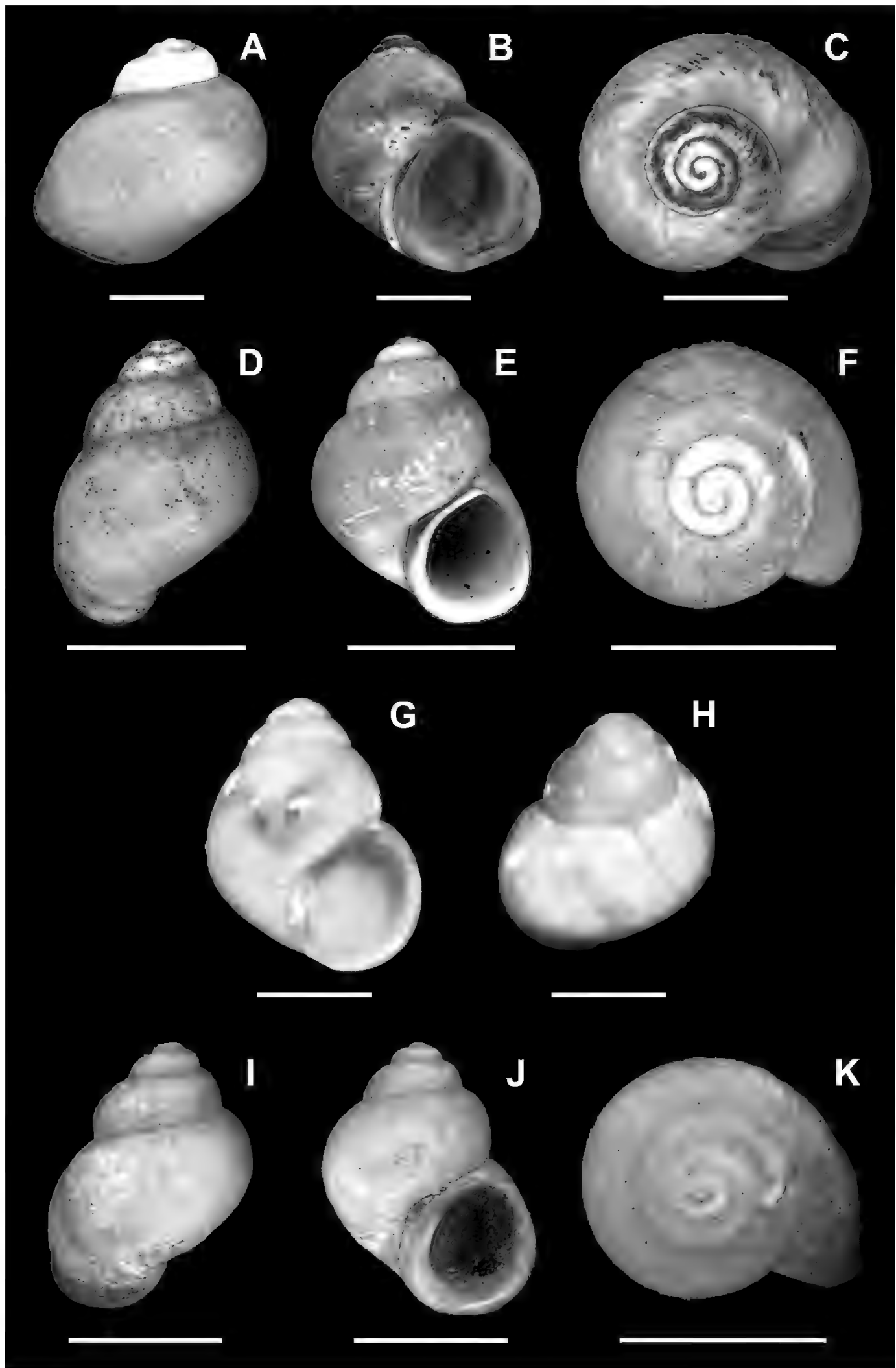
Figure 8. *Potamolithus* sp. 9 **A** (dorsal view) **B** (apertural view) **C** (apical view); *Potamolithus* sp. 10 **D** (dorsal view) **E** (apertural view) **F** (apical view); *Potamolithus* sp. 11 **G** (dorsal view) **H** (apertural view); *Potamolithus* sp. 12 **I** (dorsal view) **J** (apertural view) **K** (apical view). Scale bars: 1mm. (Photographs: LBR Fernandes).

Table I. *Potamolithus* spp. from Alto Ribeira karst area, São Paulo state, Southeastern Brazil; e, epigean, tph, troglophile, tb, troglobite; N, number of examined specimens; max. height × width (mm), height × width of largest individual (larger shell height); + present, – absent; » rivers, W caves; hom., homogenous.

Taxa	Characters							
	N	Shell shape	Max. height × width (mm)	Periostracum	Eye	Body pigment	Intestine shape	Localities
P. ribeirensis (e)	50	Globose	5.7×3.7	Dark brown	+	Black (hom.)	U-shapped (sensu Simone & Moracchioli, 1994)	≈ Iporanga
Potamolithus sp. 1 (e)	44	Fusiform	2.8×2.2	Light brown	+	Black spots along the body	Type 2	≈ Betari, Água Quente, Morro Preto
P. karsticus (tph)	27	Fusiform	2.7×1.8	Dark brown	+	Black spots along the body	Type 1 - left	≈ Calcário Branco; W Calcário Branco
Potamolithus sp. 2 (tph)	43	Fusiform	2.8×1.7	Light brown	+	Depigmented	Type 1 - right	≈ Ouro Grosso; W Ouro Grosso (close to cave resurgence)
Potamolithus sp. 3 (tph)	46	Fusiform-globose	3.6×2.3	Light brown	+	Black tentacles	Type 2	≈ Alambari; W Alambari de Baixo
Potamolithus sp. 4 (tph)	36	Fusiform-globose	2.8×1.3	Pale yellow	+	Depigmented	Type 1 - left	≈ Água Suja; W Água Suja
Potamolithus sp. 5 (tph)	68	Fusiform-globose	2.8×1.7	Pale yellow/ Translucent	+	Depigmented	Type 2	≈ Roncador; W Santana, Pérolas
Potamolithus sp. 6 (tph)	50	Fusiform	2.7×1.5	Light brown	+	Black spots between eyes	Type 1 - left	≈ Maximiano; W Casa de Pedra, Água Sumida
Potamolithus sp. 7 (tph)	15	Fusiform	2.0×1.0	Brown	+	Black stripes in the dorsal region	Type 1 - left	≈ Rio das Ostras; W Tapagem
P. troglobius (tb)	70	Globose	2.8×2.1	Translucent/White	-	Depigmented	Type 4	Ω Areias System
Potamolithus aff. *troglobius* (tb) (cited as *Potamolithus* sp. 2 in Bichuette and Trajano 2003)	82	Globose	3.2×2.3	Translucent/White	-	Depigmented	Type 4	Ω Alambari de Cima
Potamolithus sp. 8 (tb)	57	Globose	2.3×1.3	Translucent	+/-	Depigmented	Type 5	Ω Couto, Morro Preto
Potamolithus sp. 9 (tb)	35	Globose	3.9×2.9	White	+/-	Depigmented	Type 3	Ω Pescaria
Potamolithus sp. 10 (tb)	17	Fusiform	2.6×1.4	Translucent	-	Depigmented	Type 4	Ω Betari de Baixo
Potamolithus sp. 11 (tb)	30	Globose	2.4×1.7	Translucent	-	Depigmented	Type 1-left	Ω Jeremias
Potamolithus sp. 12 (tb)	17	Fusiform	1.8×1.1	Pale yellow	-	Depigmented	Type 2	Ω Colorida

Table 2. Dunn's post hoc results (p values) considering the shell size (shell height) of *Potamolithus* species from Alto Ribeira karst area, Southeastern Brazil. Gray cells, epigean species; blue cells, troglophilic species; green cells, troglobitic species; yellow cells, significant differences.

|	*ribeirensis*	sp. 1	*karsticus*	sp. 2	sp. 3	sp. 4	sp. 5	sp. 6	sp. 7	*troglobius*	aff. *troglobius*	sp. 8	sp. 9	sp. 10	sp. 11	sp. 12
ribeirensis																
sp. 1	2.38^{-14}															
karsticus	7.73^{-12}	1														
sp. 2	2.13^{-9}	1	1													
sp. 3	1.63^{-6}	1	1	1												
sp. 4	1.23^{-11}	1	1	1	1											
sp. 5	2.52^{-9}	1	1	1	1	1										
sp. 6	4.43^{-8}	1	1	1	1	1	1									
sp. 7	7.53^{-14}	1	1	0.128	0.005	1	0.009	0.016								
troglobius	2.10^{-15}	1	1	1	1	1	1	1	0.564							
aff. *troglobius*	1.18^{-11}	1	1	1	1	1	1	1	0.024	1						
sp. 8	2.03^{-26}	1	1	0.026	7.80^{-5}	1	6.94^{-5}	0.0005	1	0.168	0.0003					
sp. 9	1	3.96^{-9}	5.94^{-8}	1.59^{-5}	0.002	2.17^{-7}	5.32^{-5}	0.0002	1.84^{-10}	3.37^{-9}	2.09^{-7}	1.41^{-17}				
sp. 10	1.83^{-15}	1	1	0.062	0.002	1	0.003	0.006	1	0.298	0.009	1	1.32^{-11}			
sp. 11	2.79^{-14}	1	1	1	0.208	1	0.381	0.658	1	1	1	1	1.09^{-9}	1		
sp. 12	3.32^{-20}	0.039	0.304	0.0004	4.10^{-6}	0.022	6.04^{-6}	1.88^{-5}	1	0.003	2.18^{-5}	1	1.45^{-15}	1	0.742	
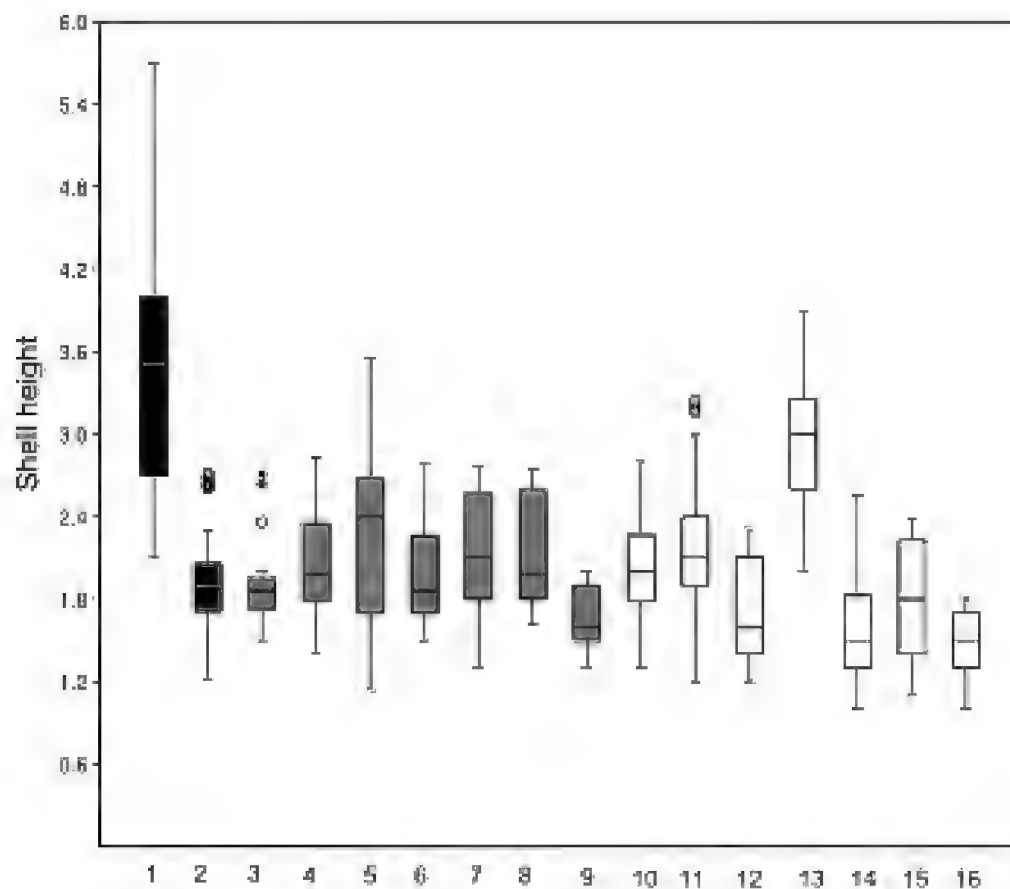
Figure 9. Boxplots showing shell heights on *Potamolithus* spp. Horizontal Bar, median; vertical bar, whiskers with minimal and maximum observations. **1** *P. ribeirensis* **2** *Potamolithus* sp. 1 **3** *P. karsticus* **4** *Potamolithus* sp. 2 **5** *Potamolithus* sp. 3 **6** *Potamolithus* sp. 4 **7** *Potamolithus* sp. 5 **8** *Potamolithus* sp. 6 **9** *Potamolithus* sp. 7 **10** *P. troglobius* **11** *Potamolithus* aff. *troglobius* **12** *Potamolithus* sp. 8 **13** *Potamolithus* sp. 9 **14** *Potamolithus* sp. 10 **15** *Potamolithus* sp. 11 **16** *Potamolithus* sp. 12. Black bars, epigean species; gray bars, troglophilic species; white bars, troglobitic species; circles, outliers; *, extremes.
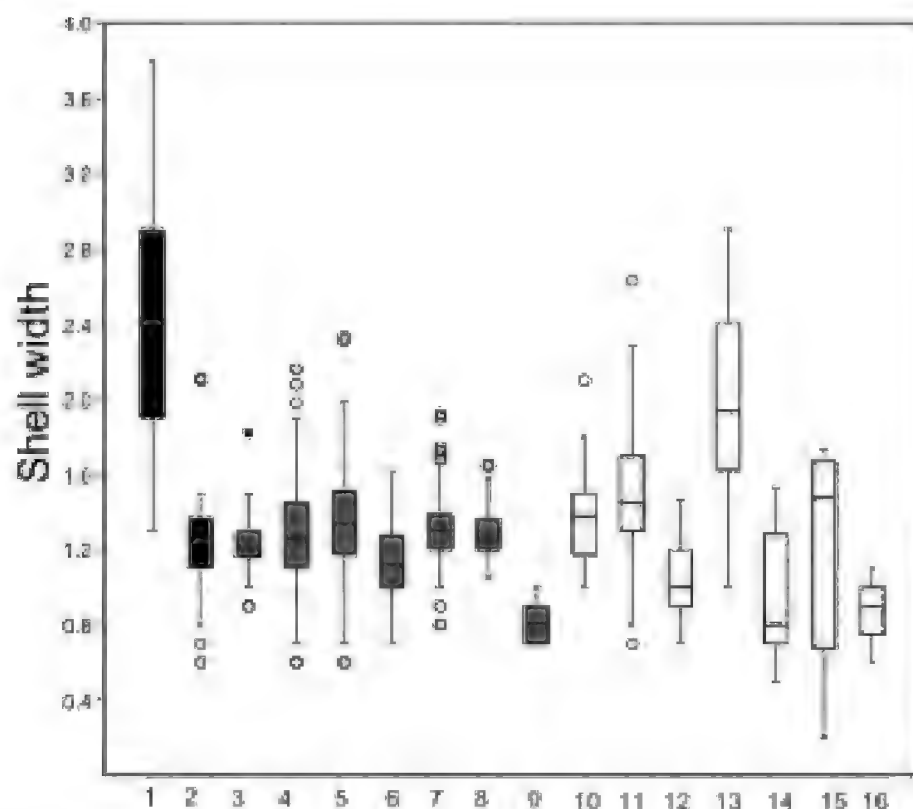

Figure 10. Boxplots showing shell widths on *Potamolithus* spp. Horizontal Bar, median; vertical bar, whiskers with minimal and maximum observations. **1** *P. ribeirensis* **2** *Potamolithus* sp. 1 **3** *P. karsticus* **4** *Potamolithus* sp. 2 **5** *Potamolithus* sp. 3 **6** *Potamolithus* sp. 4 **7** *Potamolithus* sp. 5 **8** *Potamolithus* sp. 6 **9** *Potamolithus* sp. 7 **10** *P. troglobius* **11** *Potamolithus* aff. *troglobius* **12** *Potamolithus* sp. 8 **13** *Potamolithus* sp. 9 **14** *Potamolithus* sp. 10 **15** *Potamolithus* sp. 11 **16** *Potamolithus* sp. 12. Black bars, epigean species; gray bars, troglophilic species; white bars, troglobitic species; circles, outliers; *, extremes.

Potamolithus aff. *troglobius*. In addition, there were no cases of syntopy. Considering the shell surface, SEM images showed an absence of ornamentations for *Potamolithus* studied here.

Potamolithus snails are distributed in three separate tributary basins of the Upper Ribeira River: the Betari, the Iporanga, and the Pilões river basins. In the Betari basin, these gastropods were found in its six tributaries (Água Suja, Roncador, Morro Preto, Ouro Grosso, Alambari, and Água Quente), and in 15 out of 16 visited caves (the only exception being the Córrego Seco cave). In the Iporanga river basin, representatives of this genus were recorded in two (Iporanga and Maximiano) of the four visited tributaries, and in two of the five visited caves (Água Sumida and Casa de Pedra caves), which are crossed by the Maximiano river. In the Pilões river basin, *Potamolithus* gastropods were recorded in one out of two visited caves (Pescaria cave) and there were no records in the epigean streams. Toward the northwest (Bocaina river basin, tributary of Pilões basin), *Potamolithus* specimens were recorded in a single cave (Colorida cave), among the four visited and their respective epigean reaches. To the southeast, *Potamolithus* gastropods were found in an epigean river (Rio das Ostras), and formed a troglophilic population in Tapagem cave.

The only case of a troglobitic *Potamolithus* occurring in caves from different systems was recorded in Couto and Morro Preto caves (*Potamolithus* sp. 8). However, these caves connect through their vadose zones and, during floods, there is an opportunity for dispersal between habitats.

Below we present an identification key to the *Potamolithus* from the Alto Ribeira karst area in São Paulo State:

1	Eyes absent, if present then periostracum white or translucent	**2**
–	Eyes present	**7**
2	Periostracum white, intestine type 3 ......***Potamolithus* sp. 9** (Pescaria cave) (Figure 8A, B, C)	
–	Other combination of characters	**3**
3	Intestine type 2 .... ***Potamolithus* sp. 12** (Colorida cave) (Figure 8I, J, K)	
–	Other combination of characters	**4**
4	Intestine type 5 ....***Potamolithus* sp. 8** (Morro Preto and Couto caves) (Figure 7G, H, I)	
–	Other combination of characters	**5**
5	Intestine type 1-left ...... ***Potamolithus* sp. 11** (Jeremias cave) (Figure 8G, H)	
–	Intestine type 4	**6**
6	Shell globose ......***P. troglobius* and *Potamolithus* aff. *troglobius*** (Areias system and Alambari de Cima cave) (Figure 7A–F)	
–	Shell fusiform ......***Potamolithus* sp. 10** (Betari de Baixo cave) (Figure 8D, E, F)	
7	Shell globose, periostracum dark brown, intestine U-shaped ......***P. ribeirensis*** (Iporanga river) (Figure 4A, B)	
–	Other combination of characters	**8**

8   Shell fusiform, periostracum dark brown and intestine type 1-left...............
       ....*P. karsticus* (Calcário Branco cave) (Figure 5A, B)
–   Other combination of characters...**9**
9   Shell fusiform **10**
–   Shell fusiform-globose...**13**
10   Periostracum light brown, intestine type 2 ....*Potamolithus* **sp. 1**
       (Betari, Água Quente, and Morro Preto rivers) (Figure 4C, D, E)
–   Other combination of characters..**11**
11   Mantle depigmented, intestine type 1-right................ *Potamolithus* **sp. 2**
       (Ouro Grosso cave and epigean drainage) (Figype 5C, D, E)
–   Other combination of characters..**12**
12   Periostracum   light   brown,   mantle   with   black   spots   between
       eyes..*Potamolithus* **sp. 6**
       (Maximiano river, Casa de Pedra, and Água Sumida caves) (Figure 6G, H)
–   Periostracum brown, mantle with black stripes in the dorsal region ....
       .... *Potamolithus* **sp. 7** (Ostras river and Tapagem cave) (Figure 6I, J, K)
13   Periostracum pale yellow, mantle depigmented, and intestine type 1-left.............
       Potamolithus **sp. 4** (Agua Suja cave and epigean drainage) (Figure 6A, B, C)
–   Other combination of characters..**14**
14   Periostracum light brown, mantle with black tentacles....*Potamolithus* **sp. 3**
       (Alambari de Baixo cave and epigean drainage) (Figure 5F, G, H)
–   Periostracum pale yellow/translucent, mantle depigmented............................
       Potamolithus sp. 5 (Roncador river, Santana, and Pérolas caves) (Figure 6D, E, F)

Discussion

Distribution, habitat preferences, and fragmentation: the origins of the diversity of *Potamolithus* gastropods in the Alto Ribeira karst area

With more than 60 troglobitic species, the Alto Ribeira is a high-diversity area for troglobites (*sensu* Trajano et al. 2016, Gallão and Bichuette 2018). This is among the highest genetic diversities recorded for Brazil, and is likely related to three factors: the high degree of habitat fragmentation, limited mobility leading to low capacity for dispersal, and habitat preferences.

Potamolithus gastropods prefer relatively shallow (0.6 m maximum depth) lentic locations, with clear waters, neutral to basic pH (values between 7 and 8), high conductivity (0.211 ms/cm), and relatively high temperatures (20 °C average) for Ribeira standards (Bichuette and Trajano 2003). These snails appear to be bioindicators of good quality water, since no specimen was observed in locations close to sewage discharges. Indeed, the spatial distribution of *Potamolithus* in the Ribeira Valley seems to be constrained by environmental conditions, since we did not record specimens in sites with strong currents, domestic pollution, at depths greater than 1 m, or devoid

of substrates for attachment (such as pebbles and boulders). The pH and conductivity values were high in the different caves/epigean rivers with *Potamolithus* records.

Solem and van Bruggen (1984) observed significant positive correlations between the species richness of freshwater gastropods and high values of pH, conductivity, hardness, and alkalinity on the one hand and, on the other, negative correlations with high levels of suspended particles. According to Macan (1949) and Dillon (2000), good conditions for the occurrence of operculate snails in freshwater are a large water volume, moderately warm and alkaline habitats, with little or no organic solids, and no pollution. Among truncatelloids from temperate regions, *Bithynia tentaculata* (Linnaeus, 1758) are extremely sensitive to low oxygen concentrations and avoid water with strong currents, showing a preference for backwaters (branches of rivers) with depths between 70 and 180 cm. Polluted waters, with low dissolved oxygen, are avoided by *Peringia ulvae* (Pennant, 1777) (Fretter and Graham 1962), another truncatelloid species. The troglobitic *Pseudotricula eberhardi* Ponder, 2002, from Tasmania, set up on smooth rocks that occur in small hypogean streams, preferring shallow sites (Ponder 1992).

The main tributaries of the Alto Ribeira basin in São Paulo State (left bank) cross limestone outcrops, forming several, semi-isolated micro-basins. Most of the rivers flowing across the limestone of the Alto Ribeira valley form cave systems, and almost all of them have their own troglophilic, or troglobitic, populations (Trajano et al. 2016). Moreover, insoluble rocks, such as phyllites, are intercalated with limestone outcrops (Karmann and Sànchez 1979), which probably limit subterranean dispersal (Fernandes et al. 2013). Insoluble rocks represent barriers, which may be facilitating in the isolation and speciation process. Furthermore, the fact that there are no observed cases of syntopy, points to allopatric speciation events.

In addition to the hydrogeology, another factor that may contribute to the restricted distribution observed in *Potamolithus* in Alto Ribeira is the limited capacity for dispersal due to the small size and slow locomotion of these animals, favoring isolation in confined areas. Ponder (1994) observed a high degree of endemism in mollusks in Southeast Australia and argued that limited mobility and restriction to some habitats (in that case, springs and ponds) would allow for relatively rapid speciation. The same was observed in Tasmania when epigean and cave fauna were compared (Ponder et al. 2005).

The paucity of records at higher altitudes in the Ribeira Valley was probably due to the lower temperatures, since the other variables (pH, conductivity, dissolved oxygen) were within the ranges observed elsewhere and the waters were even more pristine (Bichuette and Trajano 2003). In the Intervales karst area (PEI), despite the extensive collecting effort expended by E. Trajano and colleagues since the 1980s, *Potamolithus* specimens have been found in just one cave, and, in Espirito Santo-Caboclos area, located between PEI and Betari valley, these gastropods have been found in only two caves. The winter temperatures may drop to negative values in PEI, much lower than those observed in the Betari and Iporanga valleys located in PETAR (ca. 4 °C), which could be limiting the colonization of PEI by *Potamolithus* gastropods. It is possible that the PEI region represents the northern boundary of the genus distribution, at least in the Upper Ribeira Valley.

The mosaic distribution of character states observed encompasses not only the classic troglomorphic traits (presence *vs.* absence of eyes; body and periostracum pigmentation), but also apparently neutral characters under the hypogean selective regime (shell shape) and those with a less clear relationship with the subterranean way of life (body size and intestine shape - see below). This observation, allied to the high degree of habitat fragmentation, provides evidence for an independent origin of these species from one or more unknown epigean ancestors living in some of the main tributaries of the Upper Ribeira River. These ancestral populations would have colonized the micro-basins upstream and possibly became isolated in epigean headwaters during the dry phases of paleoclimatic cycles, originating in species such as *P. karsticus* and *Potamolithus* spp. 1 to 6). Further steps would be the colonization of subterranean habitats, with the establishment of troglophilic populations (all of the above except for *Potamolithus* sp. 1), and isolation in the subterranean realm followed by troglobitic speciation. In the case of *P. troglobius* and *Potamolithus* spp. 8 to 12, these originated from unknown epigean ancestors; we could not relate any of these morphs to the epigean and troglophilic ones based on distribution and morphology.

Morphological traits and subterranean life

It is noteworthy that troglobitic *Potamolithus* from the Alto Ribeira karst are not generally smaller than the troglophilic and epigean ones. For instance, the troglobitic *Potamolithus* from Pescaria cave reached larger sizes than all the troglophiles, as did the epigean *Potamolithus* sp. 1. On the other hand, all *Potamolithus* spp. recorded in the study area, including the epigean one from the Betari river basin and the troglobites from the Iporanga and Bocaina river basins, were smaller than *P. ribeirensis*, found exclusively in the Iporanga river. Simone and Moracchioli (1994) selected four epigean *Potamolithus* species occurring near the Alto Ribeira, in addition to *P. ribeirensis*, for comparison with *P. karsticus* and *P. troglobius*, and all of them were larger than the latter. Therefore, even without a phylogeny, we feel confident in proposing that the small size of these species is due to miniaturization.

The present data corroborate Bichuette and Trajano (2003) in that the small size of *Potamolithus* gastropods in the Alto Ribeira would be a preadaptation (in the sense of exaptation) favoring the colonization of subterranean habitats and establishment of troglophilic populations, and explains the wide distribution of these mollusks in the area.

Morphological data from *Potamolithus* spp. from the Alto Ribeira do not support the hypothesis of a strict correlation, or a single cause-and-effect relationship, between miniaturization and intestine coiling. The species reaching the largest size, the epigean *P. ribeirensis*, presents a U-shaped intestine, and the second largest one, the troglobitic *Potamolithus* sp. 9, from Pescaria cave, has a type-3 intestine, a coiling condition that is more advanced than that observed in the smaller troglobitic *Potamolithus* spp. 11 (type 1) and 12 (type 2). Other factors besides body size may affect intestine shape, for instance selection for an increase in intestine length (increase in absorptive sur-

face) as an adaptation to increase the efficiency of nutrient intake. This hypothesis is corroborated by the observed tendency of troglobites to have more coiled intestines (types 3, 4, or 5, except the aforementioned cases, *versus* types 1 or 2 in the epigean and troglophilic populations). In fact, a coiled condition of the intestine is also observed in deep-sea mollusks, and this is related to an increase in the area for nutrient absorption (Sanders and Allen 1973).

The relatively large size of *Potamolithus* sp. 9 may be a plesiomorphic trait, i.e., the retention of the non-miniaturized state present in a population that was extinct in the epigean habitat, or it may be the result of a secondary increase in size in a formerly miniaturized population (character reversal). Trajano and Bichuette (2016) hypothesized that the catfish *Ituglanis passensis* Fernández & Bichuette, 2002, from Passa Três cave in São Domingos karst area, Central Brazil, originated from a miniaturized population that vertically colonized the subterranean habitat through the epikarst (see Bichuette et al. 2015). As food input drastically increased—due to the opening of large conduits and collapse of the ceiling which then formed a typical lotic habitat with large amounts of organic matter carried into the cave—intraspecific competition without the constraints of food limitation would have selected larger sizes, resulting in a gigantic miniaturized species. There is no evidence of vertical colonization of caves by *Potamolithus* gastropods in the Alto Ribeira karst area; on the contrary, observations of epigean and troglophilic populations indicate that these organisms colonize the hypogean environment mostly through cave sinkholes and resurgences. However, the Pescaria cave is ecologically similar to Passa Três cave, and the model proposed for *I. passensis* may also account for the large size of *Potamolithus* sp. 9. The type-3 intestine in the latter corroborates this hypothesis because it seems inconsistent with the present-day conditions; apparently the cave habitat is not food-limited, and the relatively large size of the shell does not require coiling. A proper test for the two hypotheses—large size as a plesiomorphic condition *versus* character reversal—would start with a character optimization in a phylogeny of *Potamolithus* gastropods including the species of interest. Unfortunately, none is available to date.

The degree of troglomorphism, usually relative to visual structures and dark pigmentation, has frequently been used to infer the phylogenetic age of troglobitic taxa (species or higher), i.e., its time of isolation in the subterranean environment (Poulson 1963, Wilkens 1982, Trajano 1995). Therefore, *Potamolithus* spp. 8 and 9, from Morro Preto and Couto caves in the Betari micro-basin, and from Pescaria Cave in the Pilões micro-basin respectively, were isolated in the hypogean habitat later than the other troglobitic species (they would be "younger troglobites"). However, as pointed by Trajano (2007), this reasoning assumes fairly constant rates of morphological differentiation among subterranean taxa, but mosaics of character states in closely related groups indicate that different mechanisms act at different rates in each population. In addition, differences in population sizes affect rates of divergence.

It is noteworthy that three of the seven *Potamolithus* spp. that form troglophilic populations in the Alto Ribeira are characterized by a depigmented body (sp. 2 from

Ouro Grosso, 4 from the Água Suja, and sp. 5 from Roncador systems; the periostracum is also depigmented in spp. 4 and 5), indicating relaxed selection for dark pigmentation. On the other hand, all epigean and troglophilic populations have eyes, indicating a stabilizing selective pressure for maintenance of eyes in the epigean habitat.

Conservation: threats to *Potamolithus* gastropods in the Alto Ribeira karst area and the problem of over-collection

Degradation of water quality resulting, for instance, from quarrying and mining activities, poorly controlled tourism, and deforestation, especially upstream of the subterranean systems, may severely impact cave dwelling *Potamolithus* populations, since they show a strong preference for clean, well-oxygenated waters. Eberhard (1992) described the negative impacts of mining activities on cave snails from Tasmania.

The Alto Ribeira karst area in São Paulo State is mostly situated within the limits of State Parks, which should warrant protection for the aquatic gastropods endemic to this region. Unfortunately, in practice, this is not the case because Brazilian law allows for the exclusion of areas for any extension or social or economic reasons considered strategic for the State. This has happened recently with the area including the Areias resurgence in the PETAR, the locality of a differentiating population of the blind catfish, *Pimelodella kronei* (Miranda Ribeiro, 1907), a troglobitic catfish included on the IUCN Brazilian Red List (Endangered - EN category) (ICMBio 2016). Therefore, even protection by conservation units is not definitive. Moreover, the headwaters of several micro-basins (e.g., Pilões and Iporanga rivers) and the sources of the Betari river are not protected and are subject to pollution. Pollution by pesticides has been reported for the Betari river (Moraes et al. 2003)

Another important threat to the subterranean fauna is over-collection (Trajano et al. 2012). *Potamolithus troglobius* and *P. karsticus* are listed as Critically Endangered (CR) on the IUCN Brazilian Red List (ICMBio 2016) and collections must be controlled and responsible. *P. troglobius* is endemic to a single cave system (Areias) and it is an important component of the diet of *P. kronei*.

Regrettably, a loose ethical attitude of many modern researchers imbued by the "publish or perish" philosophy (Trajano 2017), allied with poor control of collections by the licensing authorities, have resulted in instances of over-collection. An illustrative example is the collection of a total of 3,325 specimens of *P. troglobius* from 12 sites during four sampling occasions using the Surber technique (Silva et al. 2015). This sampling technique is non-selective (with respect to benthic organisms) and disturbs the streambed in the area inside the sampling frame—where the rocky substrate must be cleaned and all fauna dislodged—and can result in significant impacts on the ecosystem when used without proper criteria; the huge number of gastropods collected (+ 3,000) indicates that this was the case in the Areias system. It is noteworthy that such collections were done for a study authorized by the Park authorities, in a cave

where another similarly authorized study—on the troglobitic catfish population using mark-recapture—was in progress for almost five years (Guil and Trajano 2013), in the context of one of the longest monitoring projects of a subterranean species worldwide, which started in the 1980s (Trajano 1991). Indeed, *P. troglobius* is part of the diet of the catfish *Pimelodella kronei,* the first troglobite described for Brazil collected in the late 1890s, and Areias system is the type-locality of *P. kronei.* There is no justification for such an enormous number of specimens to be collected. This cave has been studied biologically since the 1940s and was recognized as the first high-diversity spot for troglobites in 2005 (Trajano et al. 2016).

It is clear that any experimental design, and its associated sampling techniques, that result in the collection of such a huge number of individuals is a misconception, and a threat to be avoided. No scientific study, even focusing on a completely unknown species (not the case), would require such a sample size.

On the other hand, collections are indispensable because effective conservation policies are based on good biological data, and good biological data depend on careful examination of specimens by experts. There are no a priori rules regarding sample sizes, which depend not only on the objectives of the study but also on the taxon under consideration.

Only ethically oriented, experienced researchers are qualified to assess the need for collections and the scientifically acceptable minimum and maximum sample sizes. Therefore, the best way to ensure protection of biodiversity is to rely on the scientific community's self-regulation, initiatives such as the São Francisco Declaration on Research Assessment (DORA) as guidelines for refereeing processes for funding agencies, editorial policies for scientific journals, and selection of positions at universities and research centers, etc.

Conclusions

Potamolithus shows high morphological diversity in the Alto Ribeira karst area, and from 15 potential species, six are troglobitic ones, reinforcing the status of the region as a high-diversity spot for troglobites. Morphological data observed herein (and frequently related to isolation in subterranean environments) do not support the hypothesis of a strict correlation, or a single cause-and-effect relationship, between miniaturization and intestine coiling, as observed and stated for other subterranean aquatic gastropods. Relaxed selection for dark pigmentation in troglophilic species and stabilizing selective pressure for maintenance of eyes in the epigean and part of the troglophilic populations explain the morphological characters in these populations. There is evidence that epigean and troglophilic populations of *Potamolithus* colonize the hypogean environment mostly through cave sinkholes and resurgences. Finally, these species are threatened mostly by over-collection procedures, pollution of subterranean waters, and uncontrolled tourism.

Acknowledgements

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Supplementary material I

Morphometric data

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Data type: measurement

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